FREEZING RESISTANCE IN SOME NORTHERN FISHES[1]

MALCOLM S. GORDON, BEN H. AMDUR[2] and P. F. SCHOLANDER[3]

Department of Zoology, University of California at Los Angeles, Department of Chemistry, Harvard University, and Scripps Institution of Oceanography, University of California, San Diego

Scholander *et al.* (1957) described two groups of marine fishes from Hebron Fjord, Labrador, which spend long periods at subfreezing temperatures. One group (deep-water fishes) appears to survive by remaining perpetually in the supercooled state. The second group (shallow-water fishes) is exposed to subfreezing temperatures for about two-thirds of each year and survives these periods by combining supercooling with some increase in the osmotic concentration of their body fluids. The former group lives in water depths such that ice is absent even during the coldest winters. The latter group, however, lives in shallower areas and is therefore likely to encounter ice. Natural selection has presumably operated on this second group so that those fishes have survived best which have added some antifreeze to their blood, thereby reducing the possibility of their fatally freezing as a result of an accidental collision with some ice. Several species of Norwegian boreal and arctic fishes apparently also respond to subfreezing temperatures in much the same way as the shallow water fishes from Labrador (Eliassen *et al.*, 1960).

The conditions of life faced by the shallow-water fishes in Labrador in winter give rise to several questions, such as: how can these fishes tolerate any degree of supercooling at all, living as they do in close proximity to ice (supercooled nonarctic fishes freeze rapidly when touched by a piece of ice (Scholander *et al.*, 1957)); and what is the nature of the antifreeze substance? It is not NaCl. The present paper describes new observations bearing on these subjects.

Materials and Methods

In March, 1959, the present authors made a return visit to the Hebron Fjord. A small prefabricated laboratory hut was set up on the ice near Hebron settlement,

[1] These studies have been supported by contracts between the Office of Naval Research, Department of the Navy, and the Arctic Institute of North America; also by a research grant from the National Science Foundation (G8802). Reproduction of this paper in whole or in part is permitted for any purpose of the United States Government.

[2] Present address: Forsyth Dental Infirmary, 140 Fenway, Boston 15, Massachusetts.

[3] The authors' thanks for aid and cooperation are due the following people: In Labrador, Rev. and Mrs. Hettasch and Mr. and Mrs. T. Baird. At the Biological Station, Fisheries Research Board of Canada, St. Andrews, New Brunswick, Dr. J. L. Hart, Mr. L. R. Day, the crew of the station trawler "Mallotus," and the station staff. At UCLA, Dr. J. Mead, Department of Nuclear Medicine and Radiation Biology, and Dr. K. Allen, Department of Zoology. Arctic cold weather equipment and sleeping bags for the Labrador expedition were kindly furnished by the U. S. Army Quartermaster Research and Development Command, Natick, Massachusetts. Valuable technical assistance was provided by David Hall, Anita Battiste, Cynthia Rosenblum and Gordon Engel.

and large numbers of fishes were caught by jigging with hand lines through holes in the ice over 2–10 meters of water. Water temperatures were between − 1.68° and − 1.81° C. Two species of fishes were taken: the short-horned sculpin (*Myoxocephalus scorpius*) and the Fjord cod (*Gadus ogac*). The sculpins weighed 200–800 gm.; the cod weighed 400–1000 gm.

Observations made on numbers of intact, living sculpins and cod were: body temperature, measured by thermometer inserted several centimeters into the cloaca; and survival time following contact with ice, either on the outer body surface, on the gills, or in the main body muscle mass by insertion of crystals under the skin.

Other fishes were immediately taken into the heated laboratory and blood samples taken by heart puncture. Heparin was added to a few samples, but most were simply allowed to clot in plastic centrifuge tubes, then stored on ice (for a maximum of three hours) until they could be centrifuged on an electric centrifuge. Following removal of aliquots for the analytical procedures carried out in the field, the plasma or serum samples were sealed into Pyrex glass ampules, frozen and returned in the frozen state to Gordon's laboratory. Frozen serum samples were obtained from 32 sculpins and 6 fjord cod.

Analyses made at Hebron were: freezing point depression, using the method described in Scholander *et al.* (1957); electrical conductivity, measured on 1:10 dilutions of serum with an Industrial Instruments, Inc., Model RC-16B conductivity bridge calibrated against known NaCl solutions; urea plus ammonia nitrogen, measured on two sculpin samples by the Conway microdiffusion technique; glucose, measured on two sculpin samples by the glucose oxidase method; glycerol, spot-tested on several sculpin samples using the acrolein reaction.

In April, 1960, Gordon visited the Biological Station of the Fisheries Research Board of Canada, St. Andrews, New Brunswick. Sea water salinities at St. Andrews were similar to those in the study area in Labrador. Water temperatures were 3–4° C. Winter water temperatures around St. Andrews rarely are as low as 0° C.

Eighteen short-horned sculpins were used to determine the tolerance of a non-arctic population of this species to water refrigerated to − 1.5° C. Four remaining sculpins were kept at 4° C. and serum samples were carried frozen to California.

Forty-two tomcod (*Microgadus tomcod*—a close relative of *Gadus ogac*) were used in several groups for low temperature tolerance experiments involving cooling from 4° to − 1.5° over periods of 12–48 hours. Blood samples were obtained from five tomcod which survived at − 1.5° for 9½ days. Sixteen other tomcod maintained at 4° C. were also used for blood samples. The serum obtained from all of these fish was also carried frozen to California.

The serum and plasma samples were used in attempts to identify the antifreeze substance. Samples were stored frozen at about − 20° C. and gradually used for chemical analyses over a two-year period. A series of general analyses (e.g., freezing-point depression, Na, K, Cl, non-protein nitrogen) were carried out on groups of samples from each species; then, as what appeared to be promising leads developed, more specific techniques were used. These specific techniques

TABLE 1

Analytical procedures used on fish plasma and serum samples

Analysis	Procedure
I. General analyses done on whole serum or plasma:	
Freezing-point depression[a,b,c]	Cryoscopy (Ramsay and Brown, 1955)
Chloride[a,b,c]	Volhard $AgNO_3$ — SCN titration
Sodium[a,b,c]	Flame photometer on diluted samples
Potassium[a,b,c]	Flame photometer on diluted samples
Total phosphorus[a,b]	Colorimetry (Feigl, 1947, p. 317; Chen *et al.*, 1956)
Urea[a,b]	Conway microdiffusion technique (Natelson, 1957, p. 387)
II. Analyses done on samples deproteinized with trichloracetic acid or (usually) ethanol: diethyl ether (3:1, V/V), some samples also desalted, either by electrodialysis or with pyridine:	
Non-protein nitrogen[a,b,c]	Nesslerization (Natelson, p. 272)
Amino nitrogen[a,b]	Colorimetry (Natelson, p. 93)
Glycerol[a]	Resorcinol test (Jones, 1947); paper chromatography (Block *et al.*, 1958, pp. 178, 182)
Ascorbic and dehydroascorbic acids[a]	Paper chromatography as for glycerol; colorimetry (Schaffert and Kingsley, 1955)
Reducing sugars[a]	Paper chromatography as for glycerol; Folin-Wu method (Natelson, p. 205)
Non-reducing sugars[a]	Paper chromatography (Block *et al.*, p. 185)
Aldehydes and ketones[a]	Paper chromatography (Block *et al.*, p. 340)
α-diketones[a]	o-dinitrobenzene test (Feigl, 1956, p. 24)
Carboxylic acids[a]	Paper chromatography (Block *et al.*, pp. 216, 231)
Amino acids[a,b,c]	Paper chromatography (Scherbaum *et al.*, 1959)
Aromatic compounds[a]	$AlCl_3$ test (Shriner and Fuson, 1948, p. 89)
Primary alkyl amines[b]	Rimini test (Cheronis and Entrikin, 1957, p. 260); 2, 4-dinitrochlorobenzene test (Smith and Jones, 1948, p. 110)
Secondary amines[b]	Nickel-dithiocarbamate test (Duke, 1945)
Tertiary amines[b]	N-bromosuccinimide test (Cheronis and Entrikin, p. 258)
Purines and pyrimidines[a]	Paper chromatography (Block *et al.*, p. 285); ultraviolet spectroscopy (see III below)
III. Analyses done on sculpin samples deproteinized as in II, vacuum-distilled to dryness at 30 ± 5° C., then redissolved in water or various organic solvents—in some cases two or three solvent extraction stages, with vacuum distillation to dryness between stages.	
pH[a]	pH meter on diluted samples
Ultraviolet absorption spectra[a,b,c]	UV spectrometer on pH 2, 7 and 11 water extracts dried and re-dissolved in ethanol:diethyl ether (3:1, v/v)
Infrared absorption spectra[a,b,c]	IR spectrometer, on extracts dissolved in absolute ethanol, $CHCl_3$, CCl_4, CS_2 and diethyl ether in various sequences
Simpler carboxylic acids and esters, including lipids[a]	Gas chromatography of methyl esters in ethanol, diethyl ether, $CHCl_3$ and CCl_4 extracts (courtesy J. Mead)
Amino acids[a]	As under III above, also by column chromatographic fractionation with identification on paper (courtesy K. Allen)

[a,b,c]: Species on which analyses done. [a] *M. scorpius*; [b] *G. ogac*; [c] *M. tomcod*

TABLE II

Plasma concentrations in the short-horned sculpin (Myoxocephalus scorpius)

Substance	Concentration [$\bar{x} \pm$ S. E. (N)]		
	Labrador summer 4–7° C.	Labrador spring −1.7° C.	New Brunswick spring +4° C.†
Δ (mOsm/l.)	430 ± 10 (6)*	672 ± 15 (17) 775 ± 40 (6)	450 ± 5 (4)
Cl (meq./l.)	approx. 200*	234 ± 3 (6)	184 ± 2 (4)
Na (meq./l.)	—	216 ± 4 (6)	276 ± 2 (4)
K (meq./l.)	—	4.3 ± 1.7 (6)	6.4 ± 0.3 (4)
Total P (gm./l.)	—	0.55 ± 0.05 (5)	—
NPN (gm./l.)	—	1.3 ± 0.2 (5) 0.9 (1)*	1.7 ± 0.2 (4)
Urea-N (gm./l.)	—	0.4 ± 0.1 (3)	—
Amino-N (gm./l.)	—	0.15 ± 0.03 (4)	—

* Data from Scholander *et al.* (1957).
† Single pooled sample.

are summarized in Table I. This chemical identification effort was terminated with the exhaustion of the supply of samples.

RESULTS

Resistance to freezing in Labrador fishes

Data on osmotic concentration of the blood of fishes captured in Labrador in 1959 are included in Figure 1 and Tables II and III. These spring fishes were significantly more concentrated than the summer fishes studied by Scholander *et al.*

TABLE III

Plasma concentrations in codfish

Substance	Concentrations [$\bar{x} \pm$ S. E. (N)]			
	Gadus ogac (Labrador)		*Microgadus tomcod*† (New Brunswick)	
	Summer, 4–7° C.	Spring, −1.7° C.	Spring, 4° C.	Spring, −1.5° C.
Δ (mOsm./l.)	430 ± 10 (6)*	505 ± 10 (5) 790 ± 10 (8)*	440 ± 10 (14)	525 ± 5 (5)
Cl (meq./l.)	approx. 200*	243 ± 19 (3)	142 ± 2 (14)	166 ± 3 (5)
Na (meq./l.)	—	216 ± 4 (3)	231 ± 3 (14)	246 ± 2 (5)
K (meq./l.)	—	5.5 ± 1.4 (3)	5.1 ± 1.4 (14)	8.3 ± 0.3 (5)
Total P (gm./l.)	—	0.72 ± 0.03 (2)	—	—
NPN (gm./l.)	—	4.0 ± 0.2 (3)	1.0 ± 0.2 (14)	1.3 ± 0.2 (5)
Urea-N (gm./l.)	—	0.7 ± 0.1 (1)	—	—
Amino-N (gm./l.)	—	0.25 ± 0.02 (3)	—	—

* Data from Scholander *et al.* (1957).
† Analyses on three pooled samples for 4° C. fish, one pooled sample for −1.5° fish.

The temperature of the water from which these fishes were taken varied from − 1.68 to −1.81° C. The body temperatures of fresh-caught fishes (measured within 30 seconds of their removal from the water) were uniformly − 1.50° C. for each of three sculpins, − 1.50 to − 1.75° C. for five cod.

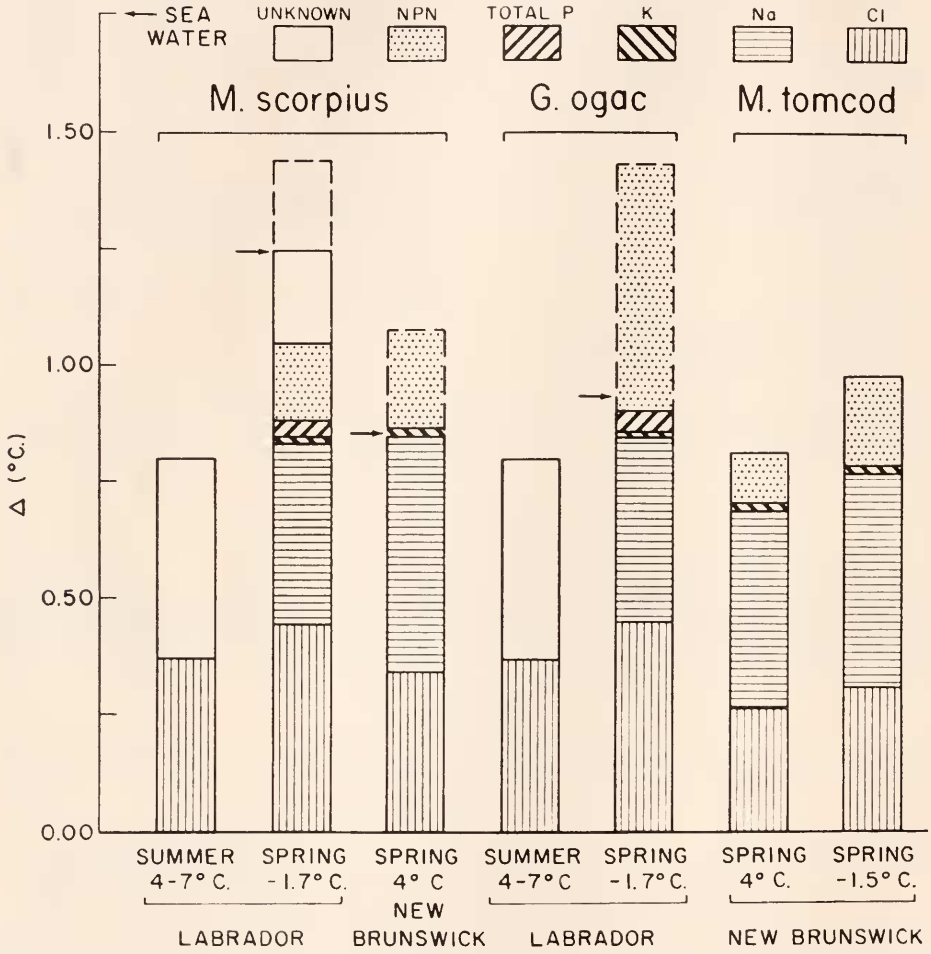

FIGURE 1. Blood serum concentrations of major constituents in groups of variously thermally acclimatized fishes of three northern species: *Myoxocephalus scorpius, Gadus ogac* and *Microgadus tomcod.* Measured concentrations converted to equivalent freezing point depression using 1 $M = 1.86°$ C. Total phosphorus (total P) and non-protein nitrogen (NPN) freezing point depressions calculated assuming one P or one N atom per molecule, respectively. Analyses for each component carried out on samples from 1–17 fishes in each group (*cf.* Tables II and III). Horizontal arrows alongside two *M. scorpius* bars and one *G. ogac* bar indicate measured freezing point depressions from 1959 (Labrador) and 1960 (New Brunswick) expeditions. Area above arrow outlined by dashes is, for spring Labrador *M. scorpius,* average measured freezing point depression from 1957 (Labrador) expedition. For spring New Brunswick *M. scorpius* and spring Labrador *G. ogac* similar areas above arrows indicate freezing point depression in excess of measured values calculated from chemical data.

It is evident that all of these fishes were supercooled by small but significant amounts—an average of 0.25° C. for the sculpins, about 0.75° C. for the cod. We therefore carried out experiments on the ease with which freezing could be induced in these fishes by seeding them with ice.

Three sculpins, damaged only very slightly by being jigged, were kept in the sea water in the fishing holes cut in the ice and observed for signs of freezing due to contact with the walls of the ice hole and with the ice particles carried over their gills by their respiratory movements. One sculpin died, showing ice crystals in one eye, within 30 minutes. The second sculpin died similarly after about one hour. The third sculpin showed ice crystals in one eye transitorily between 30 and 50 minutes after capture, then showed no further visible signs of freezing until its death after four hours. Death was unaccompanied by convulsions in these fishes.

Three cod similarly kept in an ice hole survived with no apparent freezing until observations ceased after 30 minutes. Five other cod handled similarly survived over an observation period of two hours. A sixth fish, seeded with snow and ice rubbed into a small incision in the skin of the back, died after about an hour with no visible signs of freezing. No convulsive movements were noted in any of these fishes.

Note should be made of the differences in mean osmotic concentration of the blood of the sculpins and cod taken in 1959 and those studied earlier by Scholander *et al.* We are sure that these differences are real, as the same procedures and some of the same people were involved in making both sets of analyses. The 1959 fishes were apparently significantly lower in internal osmotic concentration than were the fishes of the same species living in the same area two years earlier. Thus, while all shallow-water fishes seem to add some antifreeze to their blood during the winter, the amount added appears variable.

Resistance to freezing in New Brunswick fishes

Data on osmotic concentration of the blood of New Brunswick short-horned sculpins and tomcod are presented in Figure 1 and Tables I and II. The internal osmotic concentrations of these fishes were almost the same as the osmotic concentrations of Labrador sculpins and cod in summer, even though the New Brunswick fishes were sampled at the same time of year as were the springtime Labrador fishes. The addition of antifreeze to the blood of the sculpin, also probably the fjord cod, therefore appears to be a true response to low temperature and not simply a seasonal phenomenon. It is possible, however, that the New Brunswick fishes are physiologically different from the Labrador fishes.

New Brunswick fishes survived with difficulty when subjected to water temperatures as low as the environmental temperatures easily endured by Labrador fishes at the same time of year. At least the sculpins, however, probably can greatly improve their tolerance through gradual acclimatization.

Eighteen sculpins were transferred from sea water at 4° to sea water at − 1.5°, either directly or with some acclimation over periods longer than 12 hours. Seventeen of these froze upon coming in contact with bits of ice, whether this contact occurred immediately or after periods of up to 150 hours at − 1.50°. Only one fish, after 100 hours at − 1.5°, showed no signs of distress and did not freeze

when vigorously rubbed with bits of ice at intervals over a period of an hour. This fish froze immediately, however, when cooled to $-1.7°$ in a bucket of sea water.

It therefore seems that only a small fraction of the New Brunswick sculpin population (one of 18 fishes tested) is able to develop resistance to freezing similar to that possessed by the entire Labrador fish population. More gradual acclimation might have increased this proportion somewhat, but it seems probable that New Brunswick sculpins are physiologically different from their arctic relatives on a population basis. Somewhat similar observations by Eliassen *et al.* (1960) on supercooled Norwegian *Cottus scorpius* (probably the same species as *M. scorpius*) indicate the existence of the same situation in non-arctic eastern Atlantic fishes as well.

Though closely related, the tomcod seems to be much less resistant to low temperatures and freezing than *Gadus ogac*. Only six fishes, of 42 cooled from 4° to $-1.5°$ over periods of 12–48 hours, survived unfrozen at temperatures lower than $-1.2°$. One of these six survivors froze and died after 7 days at $-1.5°$. The other five survived until the experiment was terminated with removal of blood samples after 9½ days. It seems probable, from the freezing point of the blood of these surviving fishes (Figure 1 and Table III), that the reason for their survival was chance avoidance of any contact with the few small bits of ice which formed in their tank.

The surviving tomcod at $-1.5°$ showed three differences from controls maintained at 4°. These were: (a) volume of blood obtainable by both cardiac and caudal artery puncture about four times larger in the warm- as opposed to the cold-acclimatized fish; (b) the blood of the cold-acclimatized fish appeared to have a higher hematocrit and clotted much more rapidly at room temperature than the blood of the warm-acclimatized fish; (c) the stomachs and intestines of only the five cold-acclimatized fish were swollen with what appeared to be sea water which the fish had drunk.

These differences between the high- and low-temperature groups of tomcod, combined with the higher blood concentrations of the low temperature fishes (Table III), make it reasonable to infer that the temperature of $-1.5°$ produced an osmoregulatory disturbance in the tomcod. This disturbance may have been due to an increase over normal levels of the permeability to water of the gill membranes or integument, or may have been a result of a slowing of rates of solute excretion.

Chemical nature of the antifreeze substances in Labrador fishes

The general comment summarizing the results of the chemical analytical work carried out is that we have not been able to specifically identify the antifreeze substance in either the sculpin or the cod. We have, along with Eliassen *et al.* (1960), verified the fact that the winter (low temperature) increase in blood concentration in both forms is not due to increased concentrations of NaCl. We have also eliminated from further consideration many classes of possible compounds and have some indications as to the directions in which work should go when additional material becomes available. The sum of blood Na, Cl, and K concentrations in New Brunswick sculpins at 4° was almost the same as the sum of these same

concentrations in the blood of Labrador sculpins at − 1.7° (466 and 454 meq./l., respectively, Fig. 1 and Table II). The osmotic concentrations of these two groups of blood samples differed, however, by over 200 mOsm./l.

The picture in the fjord cod is not so clear, due to the lack of complete data from cod at higher temperatures. However, plasma Cl concentrations in winter cod were no more than 40 meq./l. higher than in summer fish. Osmotic concentrations differed by 75–360 mOsm./l.

An important biochemical difference between the short-horned sculpin and the fjord cod, a difference probably indicative of the nature of the antifreeze substance in the cod, is that shown by the non-protein nitrogen values for each form. Both species seem to have a great deal more NPN in their blood than do most other teleost fishes (NPN concentrations in the blood of many species of marine teleosts are in the range 0.04–0.73 gm./l. (Cordier and Chanel, 1958; Denis, 1922, Drilhon, 1952; Jonas and MacLeod, 1960), with only the Japanese eel in summer reaching a level as high as 1.25 gm./l. (Kawamoto, 1929)). In addition, however, the fjord cod seems to have about three times as much NPN as does the sculpin. Assuming all NPN substances in both species are in the form of molecules containing only one N atom, this fraction could supply more than enough solute to account for the wintertime increase in osmotic concentration in the fjord cod. It thus seems possible that the antifreeze of the fjord cod is a part of the NPN fraction. This is, however, not true for the sculpin, and NPN levels do not seem to vary significantly with temperature in this latter species.

Osmotically significant amounts of the compounds and groups of compounds listed in Table I were absent from the fjord cod blood samples tested. All amine tests were negative, even when samples had been treated with powdered zinc in order to reduce any oxides which may have been present (*e.g.*, trimethylamine oxide).

A point of interest concerning the fjord cod samples is the identity of the commonest free amino acids which were present. These were: aspartic and glutamic acids, threonine and monoiodotyrosine. Small quantities of samples were used in all of these analyses, so it is probable that acids present in very low concentrations were missed.

The identity of the sculpin antifreeze is presently completely obscure. There is a distinct possibility that no one compound is the antifreeze. If it is a single substance, it is apparently not a part of the NPN fraction and is probably not a phosphorus-containing compound. Other compounds and groups of compounds tested for but not detected in osmotically significant amounts are listed in Table I. Analysis of the amino acids present in sculpin blood showed alanine, methionine and taurine to be present in largest quantities.

Chemical responses to low temperatures in New Brunswick tomcod

It is interesting to note that plasma NPN concentrations in tomcod maintained at − 1.5° apparently increased by a proportionately larger amount than did the freezing point depression of the blood or the concentrations of the commonest inorganic ions. Plasma NPN levels rose about 30% while osmotic concentration increased only about 20%, chloride increased about 15% and sodium increased about 6% (Fig. 1 and Table III). The compounds involved in this increase in

concentration are unidentified. There is a striking similarity between the tomcod's response to low temperature and that shown by the fjord cod.

Discussion

The degree of supercooling occurring in all arctic fishes studied to date is quite small. However, even this slight degree of supercooling carries with it significant danger of fatal freezing if seeding with ice should occur. In order for the body fluids of the Labrador sculpins to come to thermodynamic equilibrium with the fishes' own body temperatures, approximately 20% of their free body water would have to freeze. For the fjord cod the equivalent figure would be about 40–50% of free body water.

We have no way of estimating how much, if any, of the body water actually did freeze in the Labrador sculpins and fjord cod tested for survival in the presence of ice. All we know is that these fishes did survive for periods of hours in contact with ice. There were none of the usual visible signs of freezing which always occur almost immediately in non-arctic fishes subjected to similar treatment (*cf.* Scholander *et al.*, 1957). It therefore seems probable that if there was any freezing of body fluids, it occurred slowly and probably did not spread very far through the tissues of the fishes' bodies. A similar situation apparently existed in some of the fishes studied by Eliassen *et al.* (1960).

Several theoretical alternatives seem reasonable as possible explanations for these observations. First, one might postulate that the skin, gills, etc., of the Labrador sculpins and cod are less open to penetration by ice crystals than the integuments of non-arctic fishes were shown to be by Scholander *et al.* (1957).

Second, it is possible that the skins of arctic fishes are no less penetrable by ice than are those of non-arctic forms, but that their body fluids possess special properties. One such property might be a significant slowing of ice crystal growth. The antifreeze substances themselves might confer this property, as do glycerol and other alcohols and various sugars, also some proteins in pure solutions (Lusena, 1955). Another special property might be the occurrence of larger amounts of bound water around tissue proteins, etc., than are present in non-arctic forms.

A third possibility is that neither of the above suggestions is correct, but that instead the spread of ice crystals through the bodies of our fishes was inhibited by the cell membranes of the fishes' tissues, and no damaging intracellular freezing occurred. The cell contents in this circumstance would have to tolerate some dehydration. Possible support for this idea comes from the observations of Chambers and Hale (1932) on the efficiency of frog sarcolemmae and amoeba cell membranes as barriers to propagation of ice crystals.

In both of the last two situations it would seem possible for fishes which had been seeded by a chance encounter with ice, as perhaps in an effort to escape a pursuing predator, to rid themselves of such ice as may form internally by becoming physically active enough temporarily to raise their body temperatures by a few tenths of a degree. The body temperatures we measured on the Labrador sculpins indicate that they may generally be a few tenths of a degree warmer than their environment. Observations by Britton (1924) indicate that similar small differences between body and ambient temperatures may be a year-round feature of *M. scorpius* even in non-arctic areas.

Summary

1. The occurrence of small degrees of supercooling and the presence in the blood of organic antifreeze compounds are confirmed in arctic populations of short-horned sculpins (*Myoxocephalus scorpius*) and fjord cod (*Gadus ogac*) captured at Hebron Fjord, Labrador, in early spring. The quantity of antifreeze added seems variable, however.

2. Although significantly supercooled, these same fishes were found to be very resistant to freezing even though seeded with ice. Possible explanations for this resistance are discussed.

3. Non-arctic populations of the sculpin and of the tomcod (*Microgadus tomcod*, a close relative of the fjord cod) also studied in early spring were found to lack both the resistance to freezing when supercooled and also the antifreeze substances found in the arctic fishes. Very few of the non-arctic sculpins were able to develop any resistance to freezing even after several days' exposure to arctic water temperatures.

4. The antifreeze substance added to the blood of the fjord cod is indicated to be a member of the non-protein nitrogen fraction. There is no evidence that it is an amino acid, an amine or an amine oxide.

5. The antifreeze substance added to the blood of the short-horned sculpin is also unidentified. It apparently contains neither nitrogen nor phosphorus, is not glycerol or a related alcohol and probably is not a reducing or a non-reducing sugar, an aldehyde or ketone, a carboxylic acid (lipid or other), an ester or an aromatic compound.

6. Tomcod exposed to arctic water temperatures show increases in plasma non-protein nitrogen levels similar to those which occur in the fjord cod in winter.

LITERATURE CITED

Block, R. J., E. L. Durrum and G. Zweig, 1958. A Manual of Paper Chromatography and Paper Electrophoresis. Academic Press, N. Y., 2nd ed.

Britton, S. W., 1924. The body temperature of fishes. *Contr. Canad. Biol. N. S.*, **1**: 413–418.

Chambers, R., and H. P. Hale, 1932. Formation of ice in protoplasm. *Proc. Roy. Soc. London*, **110B**: 336–352.

Chen, P. S., Jr., T. Y. Toribara and H. Warner, 1956. Microdetermination of phosphorus. *Anal. Chem.*, **28**: 1756–1757.

Cheronis, N. D., and J. B. Entrikin, 1957. Semimicro Qualitative Organic Analysis. Interscience, N. Y.

Cordier, D., and J. Chanel, 1958. Variations du taux de l'azote non protéique total, de l'azote non protéique non polypeptidique et de l'azote polypeptidique dans le serum de la Rascasse (*Scorpaena porcus* L.) sous l'influence de l'asphyxie. *C. R. Soc. Biol.*, **152**: 787–790.

Denis, W., 1922. The non-protein organic constituents in the blood of marine fish. *J. Biol. Chem.*, **54**: 693–700.

Drilhon, A., 1952. Acides amines libres des sangs des poissons: étude chromatographique. *C. R. Acad. Sci.*, **234**: 466–469.

Duke, F. R., 1945. Metallo-organic complexes in organic analysis—qualitative tests for amines. *Ind. Eng. Chem., Anal. Ed.*, **17**: 146–149.

Eliassen, E., H. Leivestad and D. Moller, 1960. The effect of low temperatures on the freezing point of plasma and on the potassium/sodium ratio in the muscles of some boreal and subarctic fishes. *Arbok Univ. Bergen, Mat-Naturv. Serie*, No. **14**: 1–24.

Feigl, F., 1947. Qualitative Analysis by Spot Tests. Elsevier, N. Y., 3rd ed.

Feigl, F., 1956. Spot Tests in Organic Analysis. Elsevier, N. Y., 5th ed.

Jonas, R. E. E., and R. A. Macleod, 1960. Biochemical studies on sockeye salmon during spawning migration. X. Glucose, total protein, non-protein nitrogen and amino acid nitrogen in plasma. *J. Fish. Res. Bd., Canada,* **17**: 125–126.

Jones, C. N., 1947. Report on the detection of preservatives and bacteriostatic agents in ampul solutions. *J. Assoc. Offic. Agr. Chemists,* **30**: 486–488.

Kawamoto, N., 1929. Physiological studies on the eel. 1. The seasonal variation of the blood constituents. *Sci. Rept. Tohoku Imp. Univ., Ser. 4,* **4**: 635–642.

Lusena, C. V., 1955. Ice propagation in systems of biological interest. III. Effects of solutes on nucleation and growth of ice crystals. *Arch. Biochem. Biophys.,* **57**: 277–284.

Natelson, S., 1957. Microtechniques of Clinical Chemistry for the Routine Laboratory. C. C. Thomas, Springfield.

Ramsay, J. A., and R. H. J. Brown, 1955. Simplified apparatus and procedure for freezing-point determinations upon small volumes of fluid. *J. Sci. Instr.,* **32**: 372–375.

Schaffert, R. R., and S. R. Kingsley, 1955. Determination of reduced dehydro- and total ascorbic acid in biological material. *J. Biol. Chem.,* **212**: 59–68.

Scherbaum, O. H., T. W. James and T. L. Jahn, 1959. The amino acid composition in relation to cell growth and cell division in synchronized cultures of *Tetrahymena pyriformis*. *J. Cell. Comp. Physiol.,* **53**: 119–138.

Scholander, P. F., L. van Dam, J. Kanwisher, T. Hammel and M. S. Gordon, 1957. Supercooling and osmoregulation in arctic fish. *J. Cell. Comp. Physiol.,* **49**: 5–24.

Shriner, R. L., and R. C. Fuson, 1948. The Systematic Identification of Organic Compounds. John Wiley, N. Y., 3rd ed.

Smith, F. J., and E. Jones, 1948. A Scheme of Qualitative Organic Analysis. Blackie, London.